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Structural and Functional Studies on Biliverdin-associated Cyanoprotein from the Bean Bug, *Riptortus clavatus*

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ABSTRACT—Structural and functional analysis were performed on biliverdin-associated cyanoprotein (CP) from the hemolymph (CP-1 to CP-4) and eggs (CPegg, identical to CP-1) of the bean bug, *Riptortus clavatus*. Isoelectric focusing analysis of purified CPegg and CP-4 revealed that they are composed of a single α subunit and a more acidic β subunit respectively. N-terminal amino acid sequencing revealed that there are six different amino acid residues between the two subunits up to the 29 cycles determined, indicating that they are encoded by different genes. Appreciable sequence similarities in N-terminal region are found between the CP subunits and several insect hexamers. By chemical cross-linking analysis these CPs were demonstrated to have hexameric structures. Two dimensional peptide mapping revealed that α and β subunits share common structures in part and that CP-2 and CP-3 are hybrid molecules bearing both α and β subunits. From these results the molecular structure of CPs was established as follows: CP-1 (CPegg)$=\alpha_6$; CP-2 $=\alpha_4\beta_2$; CP-3$=\alpha_2\beta_4$; CP-4$=\beta_6$. In addition, using ^{125}I-labeled CPegg and CP-4, *in vivo* incorporation into several tissues was examined during the nymphal-adult development. During the late phase of the 5th instar, CP-4 was sequestered preferentially over CPegg by the fat body, while after emergence both proteins were suggested to be incorporated into the newly-formed cuticle. CPegg was sequestered massively by the developing ovaries whereas no appreciable incorporation of CP-4 was observed.

INTRODUCTION

The green coloration of many plant-feeding insects, which presumably provides a protective camouflage, results from a combination of a yellow carotenoid pigment with a blue pigment, biliverdin IXγ. In most cases, biliverdin occurs as a chromophore of specic blue proteins in epidermis and/or hemolymph.

Using the eggs of a hemipteran species, the bean bug, *Riptortus clavatus* as the experimental material, we recently have purified and partially characterized a biliverdin-binding protein and designated it as cyanoprotein (CPegg) [4]. CPegg is composed of a glycosylated 76 kDa subunit rich in aromatic amino acids like arylphorin-type storage hexamers [36, 37]. This cyanoprotein is different from the biliverdin-associated proteins of Lepidoptera. These proteins either have subunit molecular masses of about 20 kDa (*Manduca sexta* [16, 33] and *Pieris brassicae* [18, 19, 35]) and are thought to be tetramers, or are members of a class of very high density lipoproteins with either dimeric (*Trichoplusia ni* [20]) or tetrameric structures (*Heriothis zea* [15]). As for the number of subunits constituting insecticyanin, some authors reported the subunit structure to be trimer [2, 13, 32]). The cyanoprotein of *R. clavatus* appears to be similar to the cyanoprotein of *Locusta migratoria* [3, 9] which has similar molecular weight, physicochemical properties, and covalently bound mannose-rich oligosaccharide chains.

The hemolymph of *R. clavatus* contains four distinct CPs (CP-1 to CP-4) which are all immunologically related to CPegg [4, 5]. In the nymphal stages these fluctuate cyclically with each molt just as has been found with the lepidopteran arylphorins [6]. In nondiapause adults, CP-1 is the predominant form and is found only in the female where it accumulates in the developing oocytes [6, 23]. From these observations, it has been suggested that CPs may function both as a storage protein and an egg yolk protein. In addition to such unique functional multiplicity, *Riptortus* cyanoprotein offers a good system to study gene regulation. Under short day conditions the adult bugs enter reproductive diapause, which is readily terminated by transferring them to long day conditions or by treatment with the juvenile hormone analog (JHA), methoprene [29, 30]. We examined this phenomenon of diapause termination at the level of CP synthesis using fluorographic analysis of female hemolymph proteins [7, 23]. Diapause female adults synthesize CP (mainly CP-4) at a low level. After diapause is terminated by JHA treatment, CP-4 synthesis is suppressed within 2 days and in turn, CP-1 synthesis is remarkably activated. These results indicate that JHA switches over the synthesis of closely

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related proteins, from CP-4 to CP-1. Assuming that CP-1 and CP-4 are the products of different genes, therefore, we can recognize this system as a suitable model for studying gene regulation by insect hormones. However, information on the relationship among these CPs is still insufficient. In the present study, experiments were conducted in order to clarify the structural and functional properties of each CP molecular species more closely and to position them in relation to other insect hemolymph proteins. In this paper we purify the hemolymph and egg CPs and show that they are all homo- or heterohexamers consisting of two distinct subunits. In addition, we report that the two subunits are considered to be encoded by different genes and that both subunits show homologies with other insect hexameric proteins. By using ^{125}I-labeled CPegg and CP-4, the functional features of the two are also examined.

MATERIALS AND METHODS

Animals

The bean bug, *Riptortus clavatus* Thunberg (Heteroptera : Alydidae) was reared at 25±1°C under long day (16L : 8D) or short day conditions (10L : 14D) through the nymphal and adult stages. The bugs enter diapause in the adult stage under the short day conditions and the females develop ovaries under the long day conditions [29, 30]. As for the nymphs, the hemolymph protein profile shows no discernible difference between nondiapause and diapause-destined animals.

Hemolymph was collected with glass capillaries from a cut in the legs or a pin-hole made in the neck membrane and diluted in phosphate-buffered saline containing several protease inhibitors as described previously [25]. Hemolymph samples were kept at −80°C until use.

Purification of CPs

CPegg was purified from newly laid eggs by gel filtration followed by ion-exchange chromatography as described previously [4]. CP-1 and CP-4 were also purified from the hemolymph of nymphs and diapause adults. Hemolymph CPs were fractionated by gel filtration using a Hiload Sephacryl-300 HR column (16×600 mm, Pharmacia). Fractions containing CPs were collected and chromatographed on an ion-exchange column (Mono-Q HR, 5×50 mm, Pharmacia). Fractions containing CP-1 and CP-4 were pooled separately and again subjected to the same ion-exchange chromatography. The CP-1 preparation obtained still showed extraneous minor protein bands, whereas CP-4 was electrophoretically homogeneous on native PAGE.

Preparation of antisera

A mixture of CP-1 to 4 was obtained as described above. A New Zealand white rabbit was immunized with the CP mixture in a manner described previously [4], and antiserum reacting to all hemolymph CPs separated. This antiserum was absorbed completely by addition of puried CPegg (α subunit) and the supernatant was used as antiserum to β subunit-specific epitopes (anti-β-specific).

Polyacrylamide gel electrophoresis (PAGE)

PAGE under non-denaturing conditions (native PAGE) and in the presence of sodium dodecyl sulfate (SDS-PAGE) were carried out as described previously [4].

Isoelectric focusing (IEF)

IEF under denaturing conditions was carried out on a 5% polyacrylamide gel slab containing 8M urea, 0.5% (w/v) Nonidet P-40 and 2.5% (w/v) Ampholine (pH 3.5–9.5, LKB) according to Görg *et al.* [14]. After focusing (4000 V×hr) at 7.5°C, the gel was stained with Serva Violet 17 (Serva) according to the method of Patestos *et al.* [31].

Immunoblotting

Immunoblotting was performed as described in a previous paper [24] except that peroxidase conjugated-goat IgG fraction against rabbit IgG (heavy and light chains specific; Cappel) was used as a secondary antibody.

Chemical cross-linking analysis

Chemical cross-linking analysis using dimethylsuberimidate (DMS) as a cross-linker was carried out according to Davies and Stark [8]. Purified CPegg, CP-4 and partially purified CP-1 were dialyzed against 0.2 M triethanolamine-HCl buffer, pH 8.5, and the protein concentration of each sample adjusted to 1 mg/ml with the same buffer. A series of 50 μl of protein samples was incubated with various amount of DMS (final conc.; 0 to 6.0 μg/μl) at 28°C for 2 hr. After incubation the reaction was stopped by addition of sample buffer (0.1 M Tris-HCl, pH 8.0 containing 20% (w/v) glycerol, 3% (w/v) SDS, and 1% (w/v) 2-mercaptoethanol) followed by boiling for 2 min. The reaction products were then analyzed by SDS-PAGE on 2.5 to 10% acrylamide gel slabs.

Analysis of amino acid composition

Amino acid composition of purified CP-4 was analyzed by an automatic amino acid analysis system (model 420H, Applied Biosystems). Tryptophan was not determined.

N-terminal amino acid sequencing

Sequences of N-terminal amino acids of CP-1 and CP-4 were determined as described by Nokihara *et al.* [27]. Nymphal hemolymph proteins were separated by native PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore) in 10 mM CAPS (3-cyclohexylaminopropanesulfonic acid), pH 11 containing 10% (v/v) methanol at a constant voltage of 70 V for 6 hr [28], and the membrane stained with Coomassie brilliant blue. The bands of CP-1 and CP-4 on the membrane were cut out. Amino acid sequencing of these CPs was carried out using a gas-phase sequencer (Applied Biosystems, Model 470A) with an on-line PTH-analyzer (Applied Biosystems, Model 120A) by direct insertion of the membrane strips into the sequencer.

Two dimensional peptide mapping (2D peptide mapping)

Radioiodinated CPs in gel slices were analyzed by 2D peptide mapping by the method of Ueno and Natori [38]. This method was first described by Elder *et al.* [11]. Hemolymph from 5th instar nymphs or crude egg extract was separated by 2.5 to 15% native PAGE and stained with Coomassie brilliant blue. Bands of CPs were cut out from the gel with a razor blade. Each gel slice (5×1×1 mm) containing about 0.5 μg of protein was dried under a heat lamp. Then proteins in the gel slices were radioiodinated in 1.5 ml Eppendorf tubes by sequential addition of 20 μl of 0.5 M Na-phosphate buffer, pH 7.5 , 300 μCi of ^{125}I (ICN, specific activity: 17 Ci/mg) in 5 μl and 5 μl of chloramine T solution (mg/ml). The tubes were kept

at 27°C for 1 hr followed by addition of 1 ml of sodium bisulfate solution (mg/ml) to stop the reaction. After 15 min the sodium bisulfate solution was removed and the gel slices were washed five times with 1 ml of 10% (v/v) methanol for 15 min for each wash. The gel slices were wrapped in nylon mesh and washed in one liter of 10% methanol for 48 hr with several changes of 10% methanol. After washing the gel slices were dried and put into 1.5 ml Eppendorf tubes containing 0.5 ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) solution (50 μg/ml in 0.05 M ammonium carbonate, pH 8.0) and allowed to absorb the solution, then homogenized thoroughly. After incubation at 37°C for 24 hr the supernatant was collected after centrifugation, lyophilized, then dissolved in 10 μl of a mixture of formic acid/acetic acid/water (25:87:887 v/v/v). Samples of up to 0.5 μl (1.5×10^5 cpm) were spotted onto cellulose-coated thin layer chromatography (TLC) plates (20×20 cm, Merk) and electrophoresed for 30 min at a constant voltage of 1000 V in the same solution used to dissolve samples, overlaid by a coolant, Solvent E (Nacalai Chemicals, Japan). After electrophoresis the TLC plates were dried and the peptides were chromatographed in a second dimension with a developing solvent system of n-butanol/pyridine/acetic acid/water (32.5:25:5:20 v/v/v/v). The plates were dried again and exposed to Fuji Super HR-S films with intensifying screens at −70°C for several hours. For densitometric scanning, spots on developed films were scanned and quantitized by an image analysis system (ACI Japan, Model TIAS-1000).

Electron microscopic observation

Purified CPegg, CP-4 and partially purified CP-1 were dissolved in 0.02 M phosphate buffer, pH 7.2 containing 0.15 M NaCl, stained negatively with uranium acetate and were observed with an electron microscope (Hitachi Model 11B).

Incorporation experiments

Radioiodination of CPs was done by Chloramine T method [17]. The reaction mixture in a total volume of 210 μl contained 120 μg protein/50 μl of phosphate buffered saline (PBS, 0.02 M sodium phosphate, pH 7.2, 0.15 M NaCl), 100 μl of 0.5 M Na-phosphate buffer pH 7.5, 0.5 mCi of $Na^{125}I$ (ICN), and 50 μl of 2 mg/ml Chloramine T in 0.05 M Na-phosphate buffer, pH 7.5. After incubation for 1 min at room temperature, 200 μl of 2 mg/ml $Na_2S_2O_5$ in H_2O was added to the reaction mixture. After 15 minutes, the reaction mixture was applied to a PD-10 column (Pharmacia) which had been equilibrated with PBS. The fractions containing excluded radioactivity were combined and stored at −80°C until use.

^{125}I-labeled CPs (c.a. 3×10^5 cpm in 1 μl) were injected into the nondiapause unsexed 5th instar nymphs and female adults. After incubation at 25°C for 6 hr, ovaries, abdominal fat body and abdominal integument (mainly cuticle and epidermis) were dissected in cold PBS, and were washed three times in PBS. The radioactivity incorporated into each tissue was counted by a γ-counter (Aloka, ARC-600).

RESULTS AND DISCUSSION

Separation of CP subunit by IEF

Hemolymph CPs (CP-1 to CP-4) are separable on native PAGE. When CP bands are cut from a stained native gel and subjected to SDS-PAGE, all CPs become indistinguishable from each other and are found to be composed of a subunit with a single apparent molecular mass of 76 kD [4]. However, the native pI of CP-1 (CPegg) (7.85) is higher than that of CP-4 (7.25) and CP-2 and 3 have pIs intermediate to CP-1 and 4. Furthermore, when bands of CP-1 and CP-4 on native PAGE are excised and subjected to rocket im-

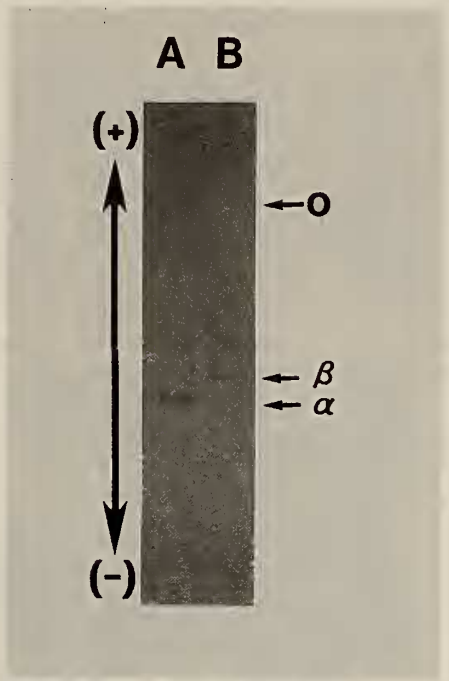


FIG. 1. Denaturing IEF of puried CPegg (A) and CP-4 (B). Samples were focused for 4000 V×hr on a 5% polyacrylamide gel slab containing 8 M urea, 0.5% (w/v) Nonidet P-40 and 2.5% (w/v) Ampholine (pH 3.5–9.5). After electrophoresis the gel was stained with Serva Violet 17. α, α subunit; β, β subunit. o: point of sample application.

TABLE 1. Amino acid composition of cyanoproteins. Data for CPegg is from [4]. Tryptophan not determined

Amino Acid	mol %	
	CPegg (α_6)	CP-4 (β_6)
Ala	6.47	7.65
Val	6.28	6.72
Leu	6.56	8.31
Ile	4.16	3.09
Pro	5.12	5.44
Met	1.48	0.54
Phe	8.96	6.89
Gly	6.16	6.93
Ser	6.65	5.78
Thr	3.99	5.04
Cys	0.00	0.00
Tyr	7.90	7.63
Asp+Asn	13.30	15.00
Glu+Gln	8.11	8.59
Lys	6.50	1.23
Arg	5.96	6.94
His	2.62	4.24

munoelectrophoresis with antiserum against CPegg, CP-1 and CP-4 form distinct precipitin lines . These facts suggest that CP-1 and CP-4 are composed of closely related subunits of the same size but different pI [5]. Separation of purified CPegg and CP-4 (see the Materials and Methods) by IEF under denaturing conditions showed that purified CPegg (identical to CP-1, see Fig. 4) was composed of a single subunit and that CP-4 was composed of a different subunit with a different pI (Fig. 1). In the previous papers we termed the putative subunits of CP-1 and CP-4 as *a* and *b*, respectively [4, 5]. Since they can be easily confused with CP-A (CP-1, 2 and 3) and CP-B (CP-4) which are separated as different precipitin lines by rocket immunoelectrophoresis [5], we therefore now name the CPegg (CP-1) subunit α and the CP-4 subunit β. The α subunit is more basic than the β subunit (subunit pIs under the denaturing conditions not determined), as is the native CPegg (CP-1) than CP-4. Some minor acidic bands other than the main band are visible for CP-4. This may be due to the uneven distribution of sialic acids in carbohydrate moiety of the β subunit.

Amino acid composition

The amino acid compositions of purified CPegg and CP-4 are presented in Table 1. The data for CPegg from [4] is also presented for comparison. Both show high aromatic amino acid (Tyr and Phe) content, 16.86% for CPegg and 14.52% for CP-4 while the Met content is low for both proteins. The content of Lys is much higher in CPegg, which may be the reason for the higher pI of CPegg.

Sequencing of N-terminal amino acids

Possible explanations for the different pIs of the two subunits are that α and β have different amino acid sequences which are encoded by different genes or that one of them is the processing product of the other. To distinguish between these alternatives, we determined the N-terminal amino acid sequences of CP-1 (α subunit) and CP-4 (β subunit) (Fig. 2). Six amino acid differences between the two subunits (indicated by dots) were seen. The amino acid sequences of the N-terminus of CP-1 and CP-4 are respectively identical to the sequences deduced from the cDNA for α and β subunits (our unpublished data). These results together with the amino

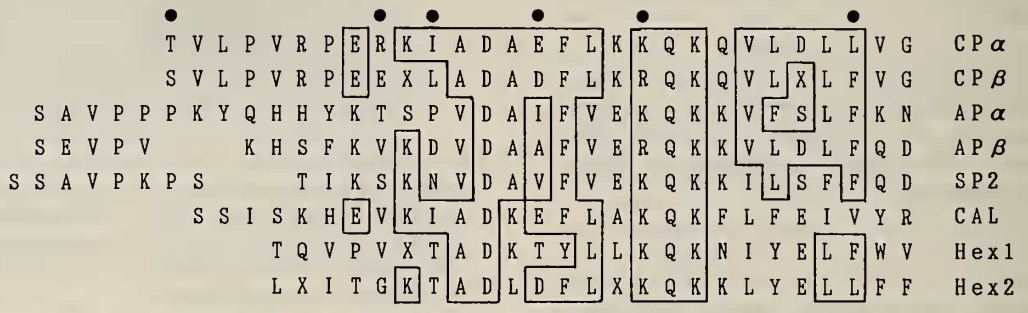


FIG. 2. Alignment of the N-terminal amino acid sequences of CPα (CP-1), CPβ (CP-4) and insect storage hexamers. Details are given under Materials and Methods. Amino acid differences between the two *R. clavatus* cyanoprotein subunits are marked with dots. Identical residues between the CP subunits and other hexamers are boxed. CPα and β: *R. clavatus* cyanoprotein α and β subunits. APα and β: *M. sexta* arylphorin α and β subunits. SP2: *B. mori* arylphorin subunit. CAL: *C. vicina* arylphorin subunit. Hex 1 and 2: subunits of *C. festinatus* storage hexamer 1 and 2. X: not identied amino acid.

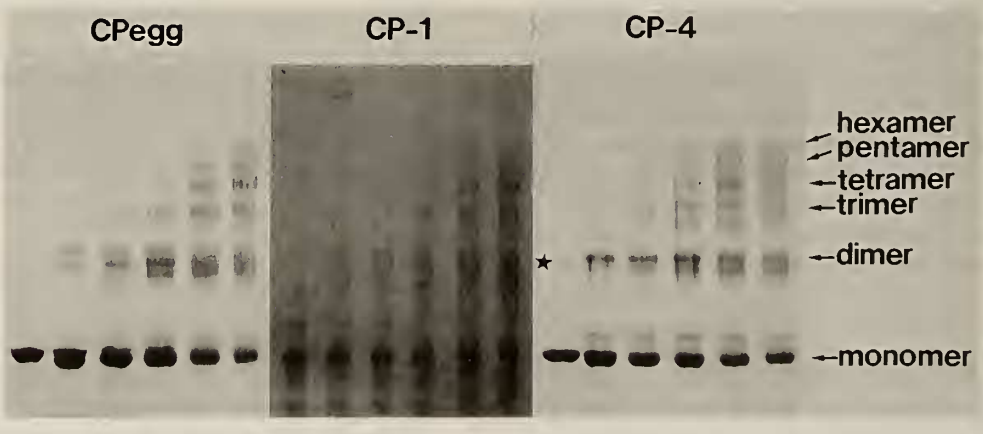


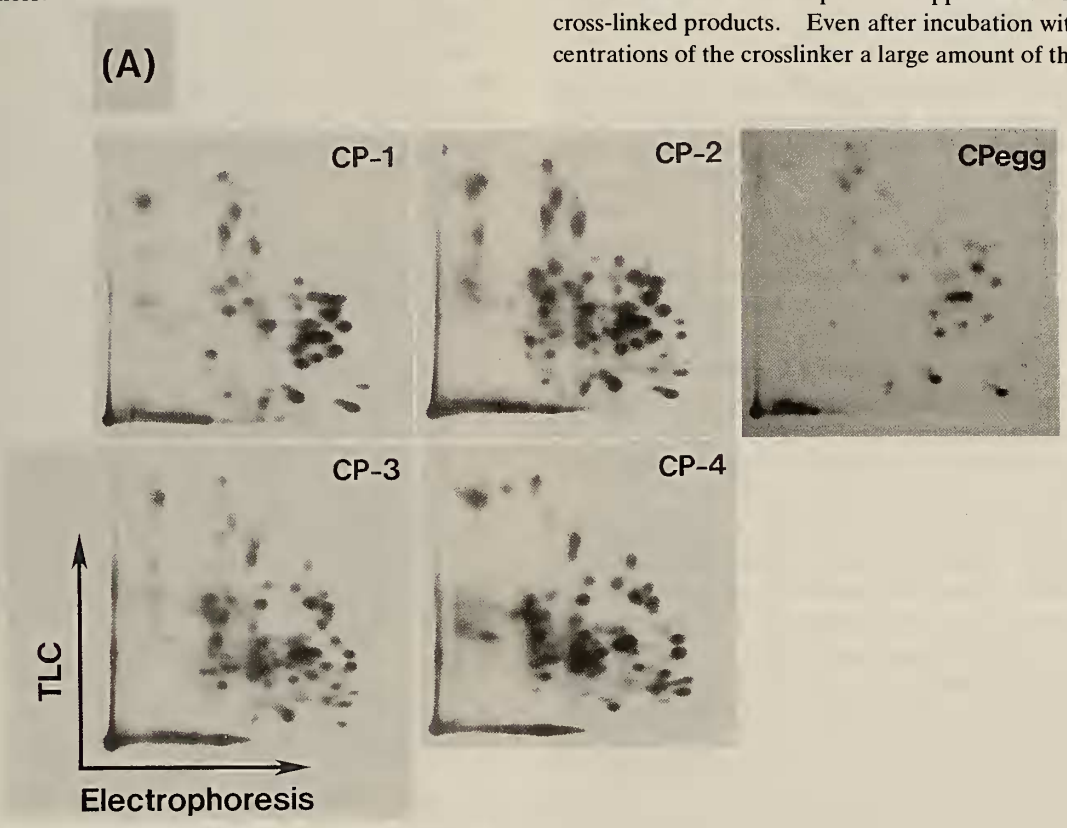
FIG. 3. SDS-PAGE analysis of chemically cross-linked products of CPegg, CP-1 and CP-4 with DMS. One microgram per one microliter of protein sample were incubated with increasing amounts of DMS. Final concentrations of the crosslinker in the incubation mixtures were 0, 0.2, 0.5, 1.0, 3.0, and 6.0 μg/μl from left to right for each protein sample. Resultant cross-linked products were analyzed by 2.5–10% SDS-PAGE. Asterisk indicates the position of the natural dimer of the β subunit which was not dissociated by SDS and 2-mercaptoethanol.

acid composition analysis confirm that the α subunit and the β subunit are products of different genes.

The two N-terminal sequences were aligned with those of several insect storage hexamers using a commercial program (Genetyx Ver. 8.0, SDC Software Development Co., Japan) (Fig. 2). As is evident here, appreciable sequence similarities in the N-terminus were found in CP subunits against storage hexamers of Lepidoptera (arylphorins of *Manduca sexta* [39], SP-2 of *Bombyx mori* [12]), Diptera (calliphorin of *Calliphora vicina* [26]) and Hymenoptera (Hex 1 and Hex 2 of *Camponotus festinatus* [22]). The results suggest that the cyanoproteins of *R. clavatus* fall in the category of insect storage hexamers.

Chemical cross-linking analysis of CPs

The CPs have a high aromatic amino acid content like arylphorin-type hexamers and show some sequence similarities to insect hexamers in the N-terminal region (Table 1 and Fig. 2). So, we examined the numbers of subunits composing native CPs by chemical cross-linking analysis. This was already announced in a footnote in the previous paper [4]. Here, we present the data. Figure 3 shows SDS-PAGE analysis of the cross-linked products of purified CPegg, CP-1 and CP-4 after incubation with increasing amounts of a cross-linker, DMS. As the concentration of DMS increased from left (0 μg/μl) to right (6 μg/μl), the cross-linked products increased in size up to the appearance of hexameric cross-linked products. Even after incubation with high concentrations of the crosslinker a large amount of the monomer



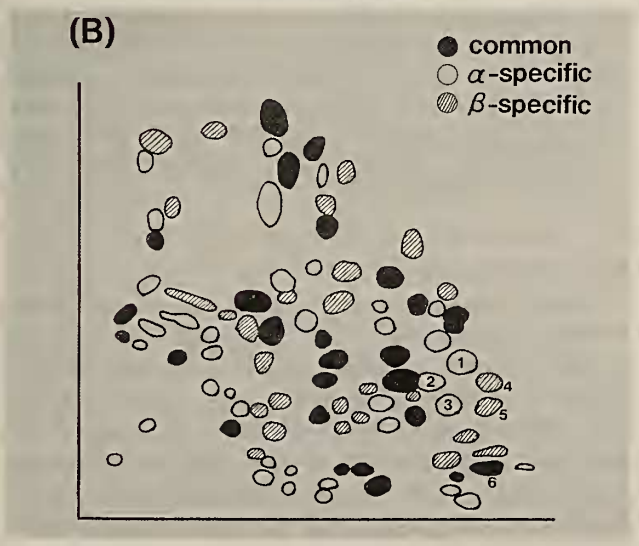


FIG. 4. Comparison of 2-dimensional tryptic peptide maps of radioiodinated CPs. CPs were separated by 2.5–15% native PAGE, and protein bands cut out from stained gels. Protein bands containing about 0.5 μg of each CPs were radioiodinated, digested with trypsin in the gel slices, and the resultant peptides extracted. Peptides were developed on cellulose plates by electrophoresis in the first dimension followed by thin layer chromatography in the second dimension, then autoradiographed. A, Maps of CP-1 to 4 and CPegg. B, A diagrammatic representation of the tryptic peptides of CPs. The spots on X-ray films were categorized into three groups as follows: spots common to both α and β subunits (solid); α subunit-specific spots (open); β subunit-specific spots (shadowed). The numbered spots were used for densitometric scanning.

still remained. This may be due to the spontaneous dissociation of the native molecules under the conditions employed for the crosslinking as discussed by Levenbook [21]. Thus, CPegg, CP-1 and CP-4 all have hexameric structures. Pore-limiting gradient PAGE analysis demonstrated that CP-2 and CP-3 have the same native molecular mass as the other CPs (not shown). Therefore, it is reasonable to consider that CP- 2 and CP-3 are also constructed by the assembly of six molecules of 76 kDa subunits.

Tryptic peptide analysis by 2D peptide mapping

In the previous paper we reported one-dimensional peptide mapping using V8 protease (without data) and discussed the subunit structures of CPs [4]. In this paper, we have employed an advanced method of 2D peptide mapping and clearly show the relationship among CP-1 to CP-4 and CPegg. CPs separated on native PAGE were radioiodinated in gel slices and digested with trypsin. The radioiodinated tryptic peptides were separated on cellulose-coated TLC plates, by electrophoresis for the first dimension and by TLC for the second dimension, then autoradiographed. The results are shown in Figure 4. As expected from the immunological relationship between CP-1 and CP-4, spots on maps of CP-1 (α subunit) and CP-4 (β subunit) could be categorized into three groups: common spots (solid); α subunit-specific spots (open); β subunit-specific spots (shadowed) (Fig. 4B). Therefore, α and β subunits have common regions as well as subunit-specific regions. The map of CPegg was identical to CP-1.

The peptide maps of CP-2 and CP-3 contained spots of all three groups. Thus, they were like superimpositions of CP-1 and CP-4. Therefore, both CP-2 and CP-3 appear to be hybrid molecules of α and β subunits. To determine the numbers of α and β subunits composing CP-2 and CP-3, the intensities of ve typical spots (1–3: α subunit-specific; 4 and 5: β subunit-specific, indicated in Fig. 4B as numbered spots) relative to one common spot (6 in Fig. 4B) were measured with an image analysis system. The relative intensities of α subunit-specific spots (1–3) in the maps decreased from CP-1 to 4 (6:4:2:0) while those of β subunit-specific spots (4 and 5) in the maps increased from CP-1 to 4 (0:2:4:6). Since CP-2 and CP-3 are also hexameric, the subunit structures of CP-2 and CP-3 were deduced to be $\alpha_4\beta_2$ and $\alpha_2\beta_4$, respectively.

According to these molecular structures, all the CP molecules are composed of even numbers of α and β subunits. As seen in Figure 3 (asterisk), small fractions of the β subunit were SDS- and 2-mercaptoethanol-nondissociable dimers in the CP-4 preparation. This natural dimer was also present in the other β subunit-bearing CPs, CP-2 and 3 (not shown). Since there is no CP molecule which has an odd number of either α or β subunits, we propose that the β subunit behaves as a dimeric form during the process of assembly of mature CP molecules.

Immunoblot analysis

CP-2, CP-3 and CP-4 were demonstrated to bear a β subunit, which is further supported here in another manner. The polyclonal antiserum obtained from a rabbit which was immunized with a mixture of CP-1 to 4 appears to be composed of three subpopulations of antibodies: antibodies recognizing epitopes common to both the α and β subunit; antibodies to α subunit-specific epitopes; antibodies to β subunit-specific epitopes. When the four CPs were analyzed by native PAGE followed by immunoblotting with this antiserum, all CP bands were visible (Fig. 5A). Then, this antiserum was absorbed completely with puried CPegg (α subunit). The absorbed antiserum (anti-β-specific) should contain solely antibodies to β subunit-specific epitopes. When a similar blot was probed with the anti-β-specific, only the bands of β subunit-bearing CPs (CP-2, 3 and 4) were detected (Fig. 5B). These results support the molecular structure, i.e. subunit composition of CP-1 to 4 mentioned above.

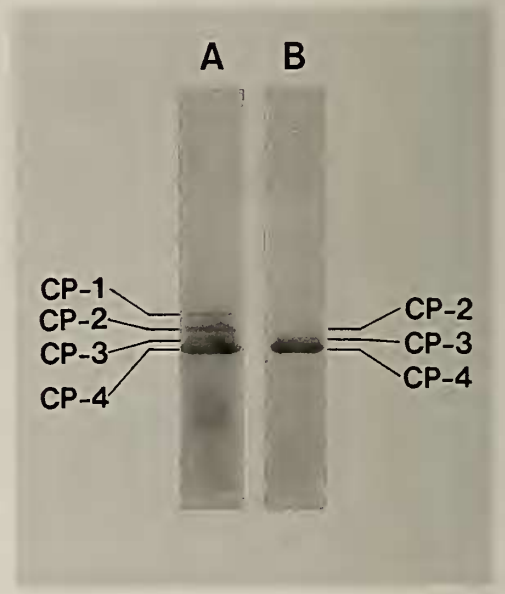


Fig. 5. Immunoblot analysis of CPs. Hemolymph protein of diapause adults was separated by 2.5–15% native PAGE, and electroblotted onto nitrocellulose. The blots were probed with (A) antiserum raised against hemolymph CPs (CP-1 to 4), or (B) absorbed antiserum to β subunit-specific epitopes (see Materials and Methods). This was followed by reaction with secondary antibodies conjugated with peroxidase.

Electron microscopy

When viewed with the electron microscope, all CPs showed hexagonal shapes touching internally to form a circle of about 130 Å in diameter (Fig. 6). Moreover, these CP molecules appear to be composed of six identical substructures, which agrees with the hexameric structures of CPs demonstrated by chemical cross-linking analysis. Some rectangular images were also observed (not shown). From these results we propose that CPs have a shape like a

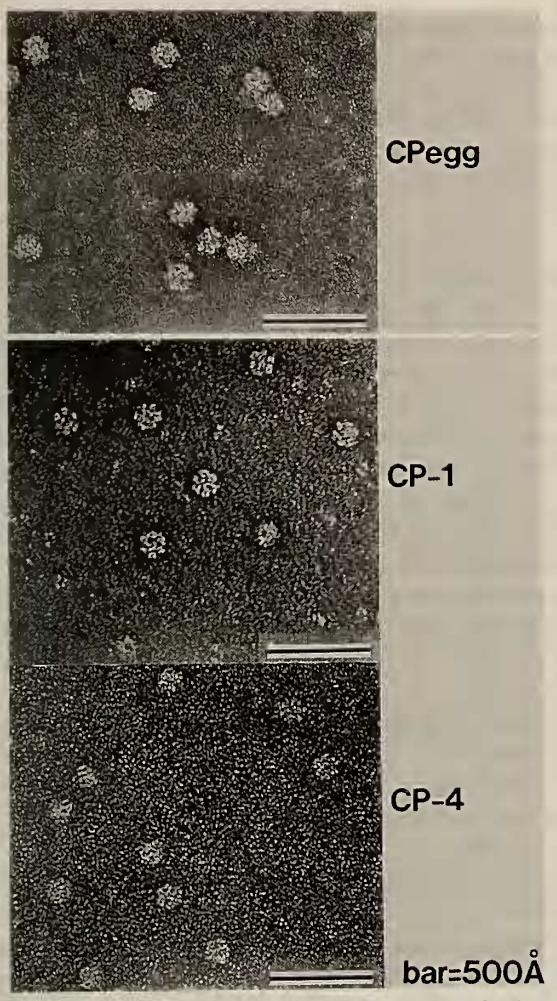


FIG. 6. Electron micrographs of CPegg, CP-1 and CP-4 stained negatively with uranium acetate. Each bar represents 500 Å.

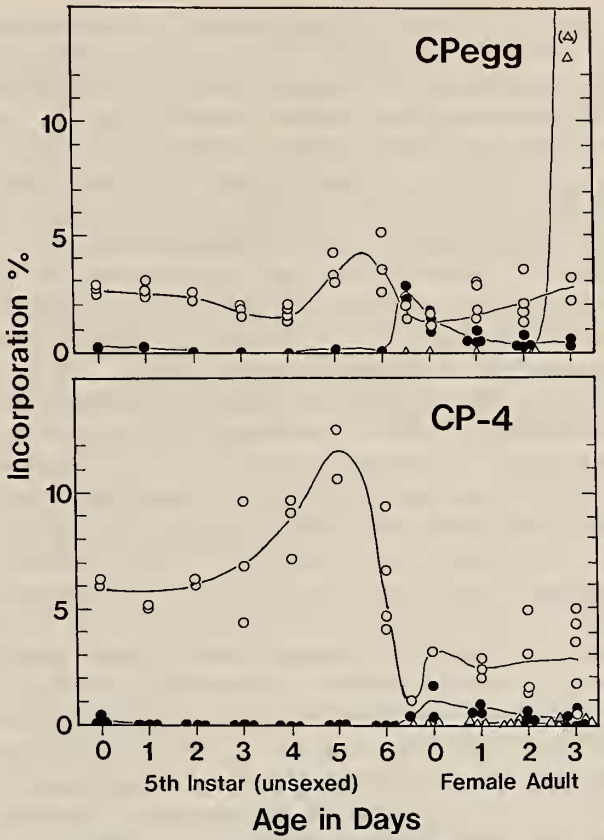


FIG. 7. Changes of CP incorporation into fat body, ovary and integument. Radioiodinated CPs were injected into unsexed 5th instar nymphs and female adults, dissected after 6 hr, and radioactivity incorporated into the tissues measured. In each experiment radioactivity of c.a. 3×10^5 cpm in a volume of 1 μl was injected per animal. Upper panel, incorporation of CPegg; lower panel, incorporation of CP-4. ○—○, fat body; △—△, ovary; ●—●, integument.

flattened hexagonal cylinder.

Incorporation of ^{125}I-labeled CPegg and CP-4 into several tissues during nymphal-adult development

Using purified CPegg (identical to hemolymph CP-1) and CP-4, we examined *in vivo* incorporation into several tissues. The purified CPs were radioiodinated by chloramine T method. The specic activities in CPegg and CP-4 preparations are 5.0×10^6 cpm/μg and 3.5×10^6 cpm/μg, respectively. The integrity of labeled CPs was checked by native PAGE and autoradiography. Electrophoretic behavior of the labeled CPs was same as unlabeled ones, and the bands showed no sign of degradation (data not shown). This confirmed that the labeled CPs still maintain their native structure.

The ^{125}I-CPegg and ^{125}I-CP-4 (3.0×10^5 cpm in 1 μl) were injected into the 5th instar nymphs (unsexed) and non-diapause female adults. After 6 hr incubation radioactivity distributing in the ovary, abdominal fat body and abdominal integument were counted following washing the tissues three times. The strength of washing (presence or absence of a detergent, Triton X-100) did not affect the radioactivity recovered. This indicates that CPs are internalized during the incubation. Results are illustrated in Figure 7. The incorporation rate is expressed as % incorporation. Through the 5th instar appreciable incorporation into the fat body was observed. The maximal rate of incorporation was demonstrated for both proteins at the final phase of the 5th instar, where the incorporation rate of CP-4 was 2.5-fold greater than that of CPegg. The rapid incorporation decreased dramatically just after adult emergence, and in turn, the incorporation into the integument was observed. The peak occurred for both proteins between day-0 and day-1 after emergence, and then declined. Thereafter, as the ovaries developed, solely CPegg was incorporated into the ovaries.

At the ultimate phase of *R. clavatus* nymphal development CP content in the hemolymph drops rapidly with a concomitant rise in the fat body content, which suggests the active sequestration of hemolymph CP molecules by fat body cells [6]. The present experiments have confirmed this hypothesis. CPegg and CP-4 were incorporated into the nymphal fat body and the maximal incorporation rate, which was about twice that at mid- instar, was observed at the final

phase of the 5th instar for both proteins. The hemolymph CP content at the final phase of the 5th instar is almost twice that of mid-instar [6]. Therefore, the specific activities of CPs injected in the late nymphs should be about one half those in the mid-instar nymphs, assuming that the labeled molecules behave in a similar manner to cold ones. Based on these values, the numbers of CP molecules incorporated at the last phase will be about 4-fold higher than those in mid-instar. Similarly, when the incorporation rate is expressed as the moles of sequestered protein, the maximal incorporation rate of CP-4 by the fat body of the last nymphal day would be 7-fold higher than that of CPegg. The question arises here whether this sequestration is driven by a nonselective or selective mechanism. We consider that some selective mechanisms at least for CP-4 sequestration other than a size barrier discussed by Duhamel and Kunkel [10] may be involved since CP-4 is shown to be incorporated at a much higher rate than CPegg. A specic receptor-mediated process found in *Sarcophaga peregrina* [38] might occur in *R. clavatus* as well. The CPs captured by the fat body are thought to be degraded without a storage process and to be utilized for constructing adult proteins because the CPs have been shown to almost disappear in the whole insect extract just after adult emergence [6]. By contrast to the fat body, the ovary sequestered exclusively CPegg (identical to CP-1) whereas no appreciable incorporation of CP-4 occurred. Moreover, hybrid molecules bearing both α and β subunits, CP-2 and CP-3 were not incorporated in the similar experiment using ^{125}I- labeled whole hemolymph proteins when examined by native PAGE and autoradiography of ovary extract after 6 hr incubation (data not shown). From these observations, the occurrence of a sorting system with high selectivity in the ovary is suggested. High pI nature and/or some structural features of protein and carbohydrate moiety of the CP-1 molecule may be involved in the ligand specificity. It was also demonstrated that CPs are incorporated into the abdominal integument of the newly emerged adults. This suggests the involvement of CPs in cuticle formation and sclerotizaition as discussed by Scheller *et al.* [34].

Conclusion

The present study confirmed that all CPs (CP-1, CP-2, CP-3, CP-4, and CPegg which is shown to be identical to CP-1) have hexameric structures composed of two types of distinct 76 kDa subunit, an α subunit and an β subunit which has a more acidic pI. Therefore, as the numbers of β subunits constituting CP molecules increase, the native pIs become more acidic. These hexamers are shaped like flattened hexagonal cylinders. There are six amino acid differences in the N-terminal amino acid sequences of the two subunits, four of which are conservative changes. Since these changes are not in a block, together with their amino acid compositions, it is reasonable to conclude that they are independent products of different genes. Moreover, the two subunits have both common and specific immunological and structural properties. The subunit structures of CPs were determined: CP-1 (CPegg)$=\alpha_6$; CP-2$=\alpha_4\beta_2$; CP-3$=\alpha_2\beta_4$; CP-4$=\beta_6$. In addition, sequencing of N-terminal regions have revealed that both subunits show appreciable sequence similarities to other insect hexamers. Therefore, we conclude that CP of *R. clavatus* is a member of hexamerin superfamily [37].

In the previous studies we obtained data suggesting its function, which, in part, has been confirmed in this study. That is, at the last phase of nymphal development, hemolymph CPs (mainly CP-4) are sequestered by the fat body and are suggested to be utilized for constructing adult proteins. Possible involvement in cuticle formation is also suggested. CP-1 in the hemolymph of the reproductive females is sequestered selectively by the ovaries and accumulates as CPegg. Among insect hexamers, CP of *R. clavatus* is more similar to arylphorin in its function during nymphal-adult development as well as in its molecular structure and high aromatic nature. These physicochemical and functional properties satisfy some of the generally accepted definitions of arylphorin type storage hexamers [21, 37]. Therefore, it is reasonable to consider that the *R. clavatus* CP is a hemipteran counterpart of arylphorin of Holometabola. Emphasis should be placed on the function of CPegg as an egg yolk protein. CPegg occupies about one third of the total egg yolk protein [4]. Although the synthesis and accumulation of storage hexamers in adults insects have been shown in orthopteran *L. migratoria* [1] and hymenopteran *C. festinatus* [22], the accumulation of storage hexamers in the egg yolk in *R. clavatus* is the only example. In this connection, we have surveyed storage hexamers in the eggs of 19 heteropteran species other than *R. clavatus* from six families, and have failed to detect them (Kamiya *et al.*, unpublished). The changes in CPs of *R. clavatus* show complicated profiles depending on stages, sex and diapause [7]. The present study has also ascertained that the various expression of CPs can be ascribed to the expression of only two genes of the CP subunits.

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